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THE EXPRESSION OF INTERLEUKIN-1B AND MIRNA-146A IN THE CEREBRAL CORTEX OF ACUTE *ESCHERICHIA COLI* MENINGITIS IMMATURE RAT MODEL

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Abstract

The main limitation to advances in treatment of bacterial meningitis and its complications is the incomplete knowledge of the pathogenesis and pathophysiology of this disease. The aim of this research is to detect the expression of interleukin (IL)-1β as pro-inflammatory cytokine and miRNA (miR)-146a as post transcriptional inflammation associated microRNA (miRNA) in the cerebral cortex of acute *Escherichia coli* (*E. coli*) meningitis immature rat model. Immature rats in the post natal day 11 (PN11) were used to construct a model of acute *E. coli* meningitis and served as controls. The expression of IL-1β and miR-146a were detected in the cerebral cortex by reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR) analysis respectively, 24 hours after bacterial inoculation. In the cerebral cortical tissue of acute *E. coli* meningitis immature rat model the IL-1β expression was significantly upregulated while the miR-146a expression was significantly downregulated. This study tried to add a new insight on the molecular basis of the *E. coli* meningitis pathogenesis at its very early stage through detecting the expression of IL-1β and miR-146a in the cerebral cortex of the infected immature rats. Consequently, modulation of the IL-1β- miR-146a axis may be a new target for treatment of acute *E. coli* meningitis.

Key words: IL-1β; miR-146a; E coli meningitis; Pathogenesis; immature rat.

Introduction

Bacterial meningitis, an inflammation of the meninges affecting the pia, arachnoid and subarachnoid space that happens in response to bacteria and bacterial products, continues to be an important cause of mortality and morbidity in neonates and children (Chang et al., 2004; De Louvois et al., 2004). *E. coli* is the most common gram-negative bacterium associated with neonatal meningitis (stoll et al., 2002).The morbidity and mortality associated with this disease has remained significant with case fatality rates ranging from 15–40% of the infected neonates while 50% of the survivors sustain neurological sequelae (Gladstone et al., 1990). The reasons for the poor outcome have been attributed to limited knowledge of pathogenesis and pathophysiology of the disease (Prasadarao, 2002). It became evident that the host immune response to the pathogen, rather than the pathogen itself, is largely responsible for the damage that results from bacterial meningitis (Weber & Tuomanen, 2007). From experimental meningitis data, it is known that areas of necrosis are mainly present in the cortex (Gerber et al., 2001). Pro-inflammatory cytokines have been shown to play a critical role in the pathogenesis of bacterial meningitis. Both tumor necrosis factor alpha (TNF-α) and IL-1β were detected in the cerebrospinal fluid (CSF) of patients with bacterial meningitis and in experimental animals (López-Cortés et al., 1993).

miRNAs are a recently discovered class of noncoding RNAs (Lagos-Quintana et al., 2001; Lee & Ambros, 2001). Typically, miRNAs are 18–24 nucleotide long, single stranded molecules that suppress the expression of protein–coding genes at the posttranscriptional level by directing translational repression, mRNA destabilization, or a combination of the two (Bartel, 2009). The role of miRNAs in inflammatory and immunologically- driven disorders is slowly being elucidated (Sonkoly & Pivarcsi, 2009). A number of miRNAs, such as the brain-enriched miR-146a have been strongly implicated in regulation of innate immune, viral, and inflammatory responses (Pogue et al., 2009; Li et al., 2010). The miR-146a is involved in innate immunity by regulating the acute inflammatory response after pathogen (bacterial rather than viral components) recognition by Toll-like receptors (TLRs) on monocytes or macrophages (Taganov et al., 2006). Nakasa et al., (2008) reported that pro-inflammatory cytokines such as TNF-α and IL-1β were reported to target miR-146a expression. The involvement of miR-146a in the regulation of inflammatory/innate immune pathways suggests that its over- or under-expression may contribute to inflammatory diseases (Sonkoly & Pivarcsi, 2009). Understanding the molecular mechanisms underlying the genesis of brain damage during bacterial meningitis will improve the treatment modalities (Gerber & Nau, 2010).

In this study we aimed to detect the expression of IL-1β as pro-inflammatory cytokine and miR-146a as post transcriptional inflammation associated miRNA in the cerebral cortex of acute *E. coli* meningitis immature rat model 24 hours after bacterial inoculation.

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Materials and methods

Experimental animals

We started the experiment with 50 male immature Sprague–Dawley rats (PN11), from Experimental Animal Center of Xiangya Medical College, Central South University, Changsha. The animals were housed in a temperature (20±2˚C), and humidity (50–60%) controlled room which was kept on an altering 12 h light–dark cycle. Animals had free access to food and water. The 50 rats were randomly divided into two groups, experimental group E (n=30) rat and control group C (n=20). They were allowed to adapt to laboratory conditions for at least one week before starting the experiments. All procedures were approved by the Institutional Animal Care and Use Committee of Central South University, Changsha.

Bacterial strain

E. coli 055/B5 was obtained from (Sigma chemical) and stored until use in skim milk at -70˚C. Stored bacteria were brought to room temperature and incubated onto trypticase soy agar containing 5% sheep's blood and sub cultured overnight (35˚C ambient air). Bacteria were suspended in sterile saline to a concentration of 0.5 McFarland standards and further diluted to 10^3 CFU/ml (Simon et al., 1991).

Induction of experimental *E.coli* meningitis

The experimental rats (n=30) were anesthetized with diethylether and placed in a stereotaxic frame for randomly intracisternal injection of 10^3 colony-forming unit (CFU) *E. coli* in 10 ul 0.85% sterile saline. While the control group (n=20), were injected with normal saline. The rats were allowed to recover in their cages with free access to food and water.

Microbiology and histology

For microbiological analysis, before rat scarification, 10 ul CSF was obtained from the experimental (n=30) and control (n=20) rats by puncture of the cisterna magna, 5 ul CSF quantitatively cultured on TTC-nutrient agar for bacterial counts, another 5ul CSF was used to count WBC . Then 10 of the infected rats were sacrificed under deep anesthesia by intra-peritoneal injection of chloral hydrate (10%, 3mL/kg) 24 h after intracisternal injection of the *E. coli* inoculums and 10 of the control rats also included. For histopathological analysis, animals were perfused via the left cardiac ventricle with 100 ml of 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Brains were immediately fixed in formalin for 24 hours and then embedded in paraffin according to standard procedures. The brains were entirely sectioned along a coronal plane. Sections were used for hematoxylin-eosin staining according to standard techniques. Morphological changes were assessed by using routine light microscopy.

Rat tissue preparation for Ribonucleic acid (RNA) isolation

After decapitation, the cerebral cortex of the other 20 infected and 10 control rats were removed quickly using RNase free instruments then all the samples were frozen on dry ice and stored at -80˚C until use.

RNA isolation

For RNA isolation, the individual frozen sample was homogenized in 1ml Trizol Reagent (Invitrogen, Carlsbad, CA, USA) for each 50 mg cortical tissue. After addition of 0.2ml chloroform, the aqueous phase was isolated using Phase Lock tubes (Eppendorf, Hamburg, Germany). RNA was precipitated with 0.5ml isopropyl alcohol, washed twice with 75% ethanol and dissolved in Nuclease-free water. The concentration and purity of RNA were determined at 260 ⁄280 nm using a nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, USA).

IL-1β expression by RT-PCR

Total cortical RNA (1µg) was reverse transcribed using RevertAid™ First Strand complementary DNA (cDNA) Synthesis Kit (#K1622, fermentas) in the presence of oligo(dT) 18 primers . PCR reactions were performed in a total volume of 25µl using Taq PCR MasterMix according manufacturer's instructions. The IL-1β primer sequences were TGGCAGCTACCTATGTCTTGC (forward) and CCACTTGTTGGCTTATGTTCTG (reverse), amplification was performed using the following conditions: 94˚C for 4min, followed by 35 cycles of 94˚C for 30s, 53.5˚C for 30s, 72˚C for 30s, followed by a final extension at 72˚C for 8min.The amplification products were visualized by electrophoresis at 90 V for 30 min in 1.5% (w/v) agarose gels and stained with ethidium bromide (0.5 µg/ml). The images were acquired by gel image system (Tanon 1600). Reactions were performed three times; values were normalized to β-actin which its primer sequences were GAGAGGGAAATCGTGCGTGAC (forward) and CATCTGCTGGAAGGTGGACA (reverse), amplification was performed using the following conditions: 94˚C for 4min, followed by 25 cycles of 94˚C for 30s, 60˚C for 30s, 72˚C for 30s, followed by a final extension at 72˚C for 8min.

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miR-146a expression by qPCR

cDNA was generated using invitrogen miRNA reverse transcription kit (Carlsbad, CA, USA) according to manufacturer's instructions. miRNA (miR-146a and the U6B small nuclear RNA gene, rnu6b) expression was analyzed using invitrogen miRNA assays , which were run on a applied bio-system 7900HT. Data analysis was performed with the software provided by the manufacturer; using 2-(ÄÄCt) method to determine relative-quantitative level of miR-146a and this was expressed as the fold-difference to the relevant control.

Statistical analysis

All experimental data were presented as mean ± SD and analyzed by Student's t test using SPSS software (version 13.0, SPSS). Statistical significance was defined as $P < 0.05$.

Results

Characterization of meningitis

24 hours after bacterial inoculation, all *E. coli* infected animals (n=30) had meningitis, as evidenced by positive bacterial titers in the CSF, while bacterial culture of CSF from saline-treated control animals (n=20) showed negative result (Fig1A). CSF leukocyte counts showed significant difference between bacterial infected animals and those treated with saline $3780 \pm 1123 \times 10^6$/L versus $16.7 \pm 6.23 \times 10^6$/L ($p<0.05$). For histopathologic studies, we found that in some of the bacterial infected animals (n=10), the granulocytes were seen in the subarachnoid space and they appeared as "red neurons" which indicate the neuronal death compared to the normal histopathological findings in the 10 control rats (Figure 1B).

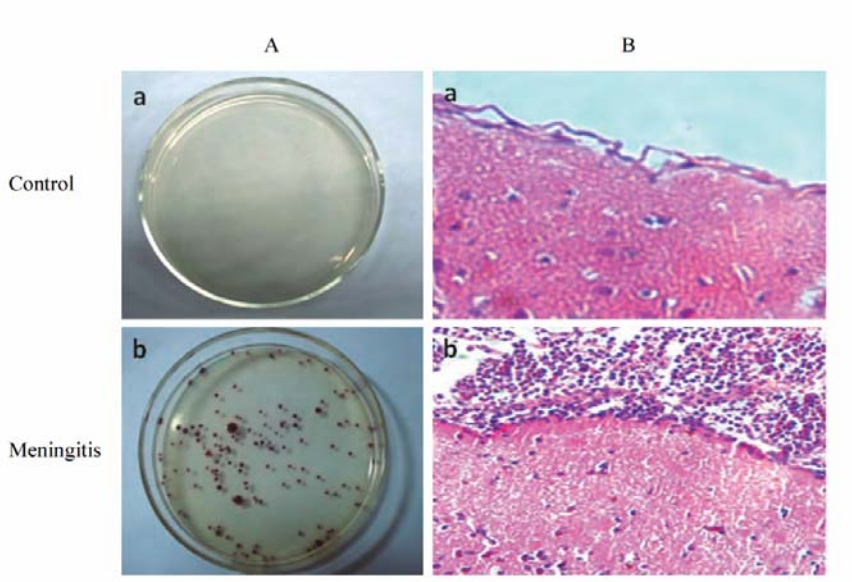

Figure 1A: Positive bacterial titers in the CSF obtained from *E. coli* infected immature rats (n=30) compared to negative results of bacterial cultures for CSF from control immature rats (n=20).

Figure 1B: Histopathologic study showed that the granulocytes were seen in the subarachnoid space and they appeared as "red neurons" in meningitis rats (n=10) compared to control rats (n=10), representing the neuron death.

IL-1β expression was upregulated in meningitis model

IL-1β expression was significantly upregulated in the cerebral cortical tissue of acute *E. coli* meningitis immature rat model (n=20), 24hours after bacterial inoculation, with mean = 0.266 ± 0.035 compared to the control rat (n=10) mean = 0.081± 0.01. The IL-1β expression was normalized to that of β-actin (Fig 2A, 2B; 2C).

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miR-146a expression was downregulated in meningitis model

miR-146a expression was significantly downregulated in the cerebral cortical tissue of acute *E. coli* meningitis immature rat model (n=20), 24 hours after bacterial inoculation, with mean = 0.5 ± 0.1 compared to the control (n=10) mean =1. The miR-146a expression was normalized to that of the U6B small nuclear RNA gene (rnu6b) (Figure 3).

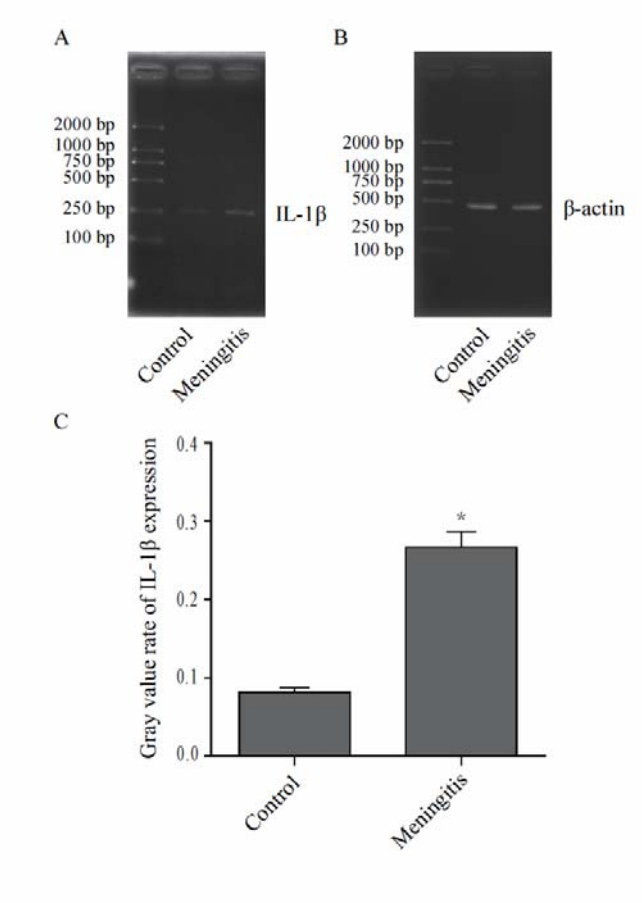

Figure 2: RT-PCR expression of IL-1β in cerebral cortical tissue of acute *E. coli* meningitis immature rats (n=20) compared to the control (n=10). (A&B) relative expression of IL-1β mRNA bands to β-actin detected by RT-PCR. (C) IL-1β was upregulated in the cerebral cortex of acute *E. coli* meningitis immature rats. *P < 0.05 compared with control.

Discussion

Despite the effectiveness of current antibiotics in clearing bacteria from the CSF, bacterial meningitis continues to cause significant morbidity and mortality worldwide (Sellner et al., 2010). Its transient or permanent neurological sequelae include deafness, epilepsy, mental retardation and impairment of motor and sensory functions arising in up to a third of survivors (Aronin et al., 1998). Since the amount of inflammation in the subarachnoid space correlates with outcome of disease, it is important to understand details of the inflammatory cascade triggered during meningitis in order to design new therapeutic agents.

The present study focused on the expression of IL-1β as pro-inflammatory cytokine and miR-146a as inflammation associated miRNA in the cerebral cortex of acute *E. coli* meningitis immature rat model (PN11) which corresponds to the term human brain (Vannucci et al., 1999), our results revealed that IL-1β was significantly upregulated while miR-146a was significantly downregulated. We are the first to study miR-146a expression in this animal model trying to add a new insight in understanding the pathogenesis of this disease.

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The pathogenesis of bacterial meningitis is determined by several factors including bacterial load, production of pro-inflammatory cytokines such as TNF-α and IL-1β, increased permeability of blood brain barrier (BBB), and infiltration of

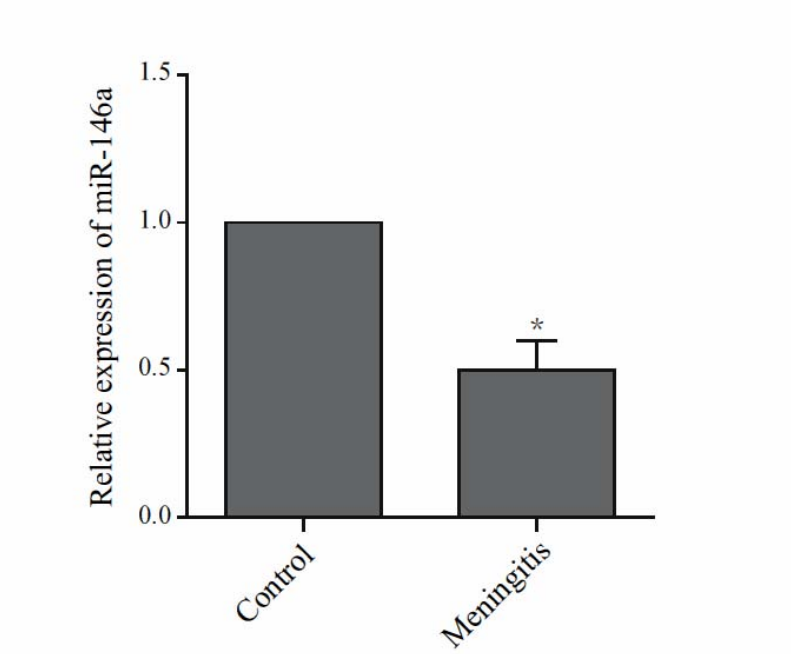

Figure 3: Quantitative real-time PCR expression of miR-146a is downregulated in the cerebral cortex of acute *E. coli* meningitis immature rats (n=20) compared to control (n=10). *P < 0.05 compared with control.

inflammatory neutrophils. The pathophysiologic sequelae of meningitis that result from the interaction between the bacteria and the host constitute a complex cascade (Tsao et al., 1999).

The presence of cytokines, such as TNF-α, IL-1β, and IL-6 in the CSF, and blood are hallmarks of the pathogenesis of neonatal meningitis (Low et al., 1995). IL-1β was first related to meningeal inflammation when it was shown that intracisternal administration of homologous IL-1β in rabbit's increased CSF white blood cell counts (Ramilo et al., 1990). Increased CSF concentrations of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-8 and the anti-inflammatory IL-10 are characteristic for bacterial meningitis (Koedel et al., 2002). TNF-α, IL-1β,and IL-6, the major early-response cytokines, trigger a cascade of inflammatory mediators including other cytokines, chemokines, arachidonic acid metabolites, reactive nitrogen and oxygen intermediates and proteases (Leib et al., 2001; Leib et al., 2003). In our study, the significant upregulation of IL-1ß in the cerebral cortex of acute *E. coli* meningitis immature rat model supports the role of this inflammatory cytokine in the pathogenesis of this disease. The results of qPCR detected significant downregulation of miR-146a in the same animal model, putting this inflammation associated miRNA as a potential candidate to understand the pathogenesis of this disease at the posttranscriptional level

Not only upregulation of miR-146a but also its downregulation can be associated with inflammation, miR-146a is found to be downregulated in Systemic lupus erythematosus, a classic autoimmune disease (Tang et al., 2009). The expression of miR-146a in both lumbar dorsal root ganglia (DRG) and spinal dorsal horns significantly decreases in animals with osteoarthritis (OA) knee joint pain compared to sham controls (Li et al., 2011). In atopic eczema, a chronic inflammatory skin disease with the dominance of Th2 type inflammation in the acute phase, miR-146a levels were unchanged compared with healthy skin (Williams et al., 2008). Loss or down-regulation of miRNA expression may be due to mutation, epigenetic inactivation, aberrant processing or transcriptional down-regulation (Calin et al., 2006).

Further researches are needed to study the mechanism of downregulation of the miR-146a in the acute stage of *E. coli* bacterial meningitis and the challenge now is to understand the details of its biological function in this animal model.

Conclusion

In conclusion, this study demonstrated upregulation of IL-1β as pro-inflammatory cytokine and down regulation of

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miR-146a as post transcriptional inflammation associated miRNA in the cerebral cortex of acute *E. coli* meningitis immature rat model 24 hours after bacterial inoculation. Consequently, modulation of the IL-1β- miR-146a axis may be a new target for treatment of acute *E. coli* meningitis.

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